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Dachs et al., Oncology research 1997, 9 (6-7), p313-25.

Putnam, Am. journal of health-system pharmacy:AJHP: official journal of the american society of health-system pharmacists, jan. 15, 1996, 53 (2) p151-60.

Marschall et al., cellular and moecular neurobioloogy, oct. 1994, 14 (5), p523-38.

9045679

Leopold et al., blood, apr. 15, 1995, 85, 8, p2162-70.

Kiehntopf et al., embo j. oct. 3, 1994, 13, 19, p4645-52.

konopka et al., faseb j. 11 (9), ;a1090, 1997

masuda et al, proceedings of the am. asso. for cancer research annual meeting 37, 0, p353-354, 1996.

eckstein, ciba foundation symposium, 1997, 209, p207-12.

SNYDER ET AL., BIOLOGY OF BLOOD AND MARROW TRANSPLANTATION-- JOURNAL OF THE american society for blood and marrow transplantation, oct. 1997, 3, 4, p179-86.

kitajima et al., j. ob biological chemistry, oct. 24, 1997, 272, 43, p27099-=106

gu et al., molecular and cellular biochemistry, jul. 1997, 172, 1-2, p47-57.

castanotto et al., methods in molecular biology, 1997, 74, p429-39.

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Exogenous Cellular Delivery of Ribozymes and Ribozyme Encoding DNAs

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1. Introduction

To examine the effects of a ribozyme in vivo, a major obstacle to overcome is the delivery of these molecules into the cells. A serious drawback of exogenous delivery is that the inhibitory effects of the nucleic acids so delivered are transient and require repeated administrations. Despite this, exogenously delivered molecules can incorporate chemical modifications, which increase stability, although these modified bases and sugar-phosphate backbones can contribute to toxicity of the molecules. Further investigation is needed to establish whether the advantages of chemical modifications will overcome the general disadvantages of exogenous delivery.

RNA synthesized in vitro by T7 RNA polymerase can also be exogenously delivered. An advantage of this approach is that the ribozyme can be inserted in larger transcripts containing structural features (stem loops), which confer stability. A disadvantage is that these extra sequences can negatively affect the catalytic activity of the ribozyme *(1)*.

Many techniques have been developed to introduce functional, naked, DNA, and some of these may also be applicable to the delivery of synthetic RNA:

1. DNA is complexed with various compounds (e.g., polylysine) or to lipophilic groups *(2–4)*, which increase cellular uptake.
2. DNA can be complexed with receptor ligands for specific targeting and localization to defined types of cells *(5)*.
3. Conjugates of DNA and lipophilic derivatives can have higher antiviral activity as shown in the case of HIV-1 *(4,6)*.

These procedures introduce nucleic acids into the cytoplasm, but access to the nucleus is remarkably poor, as a consequence of the degradation of the

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nucleic acids in the cytoplasm or endocytotic vesicles, and because of the physical barrier of the nuclear membrane. Some delivery techniques address this issue by including non-histone nuclear proteins *(7)*, phage particles *(8)*, or adenovirus protein in the nucleic acid-containing complex *(9)*. These proteins can mediate to some extent the migration of nucleic acids to the nucleus either by protecting the DNA from degradation or by facilitating the transfer through the nuclear membrane. RNA molecules are extremely sensitive to degradation in the media and require protection from serum ribonucleases. To date, the most commonly utilized technique for delivering presynthesized RNA molecules into cultured cells is via liposome encapsulation.

Exogenously synthesized ribozymes, whether chemically or biochemically synthesized, can be delivered to cells in culture via cationic liposomes. Ribozymes delivered in this manner can be used to scan a target to assess the most vulnerable site and can also be used for therapeutic applications. Although it is possible to determine directly the accessibility of a particular sequence in vivo *(10)* or in cell extracts, it requires intensive labor. Thus, accessible regions of the target should be found empirically by designing separate ribozymes to target several potential sites and testing their relative effectiveness.

This chapter summarizes protocols for in vitro transcription of ribozymes and subsequent in vitro cleavage reactions, which can be used to select efficient ribozymes. A protocol for encapsulation and delivery of nucleic acids follows. Finally, promoter systems, viral vectors, and in vivo ribozyme cleavage assays are discussed.

2. Materials

2.1. In Vitro Transcription

1. Template DNA: a linearized plasmid containing ribozyme or substrate sequences downstream of a T7 promoter, or synthetic DNA equivalents.
2. 400 m*M* Tris-HCl, pH 7.5.
3. 100 m*M* $MgCl_2$.
4. 100 m*M* NaCl.
5. 100 m*M* DTT.
6. 5 m*M* NTP solution: 5 m*M* of each ATP, CTP, GTP, UTP.
7. T7 RNA polymerase.
8. RNase-free DNase (e.g., Boehringer, Indianapolis, IN).

2.2. In Vitro Ribozyme Cleavage Reactions

1. Ribozyme: chemically synthesized or in vitro transcribed as in Section 3.1.
2. Substrate: chemically synthesized or in vitro transcribed as in Section 3.1.
3. 20 m*M* Tris-HCl, pH 7.5.
4. 500 m*M* KCl.

5. 100 m*M* $MgCl_2$.
6. Gel loading buffer: formamide containing 0.1% xylene cyanol, 0.1% bromophenol blue, and 20 m*M* EDTA.
7. 7 *M* Urea, polyacrylamide gel and running buffer (usually 1X TBE: 89 m*M* Tris-borate, pH 8.3, 2 m*M* EDTA).

2.3. Cationic Liposome Encapsulation of Ribozymes and Delivery

1. Vortex mixer.
2. CO_2 incubator.
3. Laminar flow hood.
4. Lipofectin (Gibco/BRL [Grand Island, NY] or other cationic lipid analog reagents): the reagent consists of 0.5 mg/mL DOTMA and 0.5 mg/mL DOPE in sterile water (*see* Note 1).
5. Opti-MEM I (Gibco) medium.
6. Serum (dependent on cell lines).
7. DMEM high glucose (Irvine, Santa Ana, CA).
8. 1X PBS (Irvine).
9. Fungi Bact (Irvine).
10. Pen-Strep (Irvine).
11. Fungizone (Irvine).
12. β-Mercaptoethanol.
13. Sodium pyruvate (Irvine).
14. Trypsin (1X) (Irvine).
15. Sodium bicarbonate (7.5%) solution.
16. 200 m*M* L-glutamine.
17. Plasmid DNA containing ribozyme gene transcriptional unit.
18. RNA produced from in vitro transcription or chemically synthesized.
19. Chemically synthesized ribozyme.
20. Polystyrene tubes, sterile, 17 × 100 mm (FALCON, Franklin Lakes, NJ).
21. 60 mm Culture dish (Costar, Cambridge, MA).

3. Methods

3.1. In Vitro Transcription

RNA molecules may be enzymatically synthesized using RNA polymerases. T7, T3, and SP6 RNA polymerases are commonly employed. The DNA templates encoding the ribozyme and substrates either derive from linearized, plasmid DNA, or synthetic oligonucleotides harboring the ribozyme (or substrate) sequence. In the case of plasmids, the DNA bearing the ribozyme encoding gene (or the substrate target for the in vitro assays) should be cloned in a transcriptional unit and inserted as close to the RNA polymerase promoter as possible. The plasmid should be linearized immediately downstream of the ribozyme (or substrate) sequence to minimize the amount of vector-derived flanking sequences in the transcripts.

1. In a 1.5 mL microcentrifuge tube mix:
   50 n*M* template DNA;
   40 m*M* Tris-HCl, pH 7.5;
   6 m*M* $MgCl_2$;
   5 m*M* NaCl;
   10 m*M* DTT;
   0.5–1 m*M* each of ATP, CTP, GTP, UTP; and
   10–20 U of T7 RNA polymerase.
2. Incubate at 37°C for 1–3 h.
3. Remove the template DNA by a brief treatment with 1 µL of RNase-free DNase (1 µg/µL), and purify by extraction and precipitation (*see* Notes 2–4).

3.2. In Vitro Ribozyme Cleavage Reactions

1. Heat to 90°C for 2 min two separate tubes containing either the ribozyme or substrate (^{32}P-labeled target) in a solution of 20 m*M* Tris-HCl, pH 7.5, and 0–140 m*M* KCl (or NaCl). The volume of the reactions and the concentration of ribozyme and substrate can vary. A noncleaving mutant version of the ribozyme should be used as a control.
2. Renature the samples for 5 min at the temperature chosen for the cleavage reaction (37–55°C) in presence of 10 m*M* $MgCl_2$.
3. Mix different amounts of ribozyme and target (depending on the purpose of the analysis, either equimolar, excess of ribozyme, or excess of target) at the desired temperature. Different time-points should be taken from 0–3 h (*see* Note 5).
4. Stop the reactions by adding an equal volume of formamide gel loading buffer.
5. Denature the samples by heating to 90°C for 2 min, and analyze the cleavage products on a 7 *M* urea, polyacrylamide gel. Values for k_{cat}/K_m can be determined by incubating a constant concentration of substrate (around 1 n*M*) with increasing excess of ribozyme for a constant time. The k_{cat}/K_m value is derived using the equation

$$-\ln(\text{Frac S}) / t = k_{cat}/K_m * [\text{ribozyme}] \quad (1)$$

   where Frac S is the fraction of remaining substrate and t is time *(11)*.

3.3. Cationic Liposome Encapsulation of Ribozymes and Delivery

Liposomes are comprised of one or more concentric phospholipid bilayers (which can incorporate lipid-soluble substances) surrounding an aqueous compartment that can incorporate water-soluble substances. Size and lipid composition can vary, and different liposomes exhibit different characteristics as in vivo delivery systems.

- Negatively charged lipids can increase the efficiency of cellular uptake; saturated lipids and the presence of cholesterol can increase liposome stability.
- Liposomes can be covalently attached to antibody molecules, resulting in specific binding to cellular antigens *(12)* and allowing specific targeting to different types of cells.

- pH-sensitive liposomes, on exposure to the low-pH environment of the endosomes, fuse with the endosome membranes.
- Immunoliposomes (pH-sensitive liposomes conjugated to monoclonal antibodies) can be successfully targeted to cell-surface receptors in vitro and in vivo *(13)*.
- The primary mechanism for cellular uptake of liposomes seems to be endocytosis *(14)*. Once in the cytoplasm, the liposomes are degraded, and the nucleic acids contained inside are released.

1. Plate exponentially growing cells in tissue-culture dishes at 5×10^5 cells/well, and grow overnight in a CO_2 incubator at 37°C to 80% confluency.
2. Dilute the presynthesized DNA or RNA molecules from Section 3.1., and the Lipofectin reagent (BRL) with Opti-MEM I (Gibco) medium. The amounts of nucleic acids and liposome suspension need to be optimized for each cell type.
3. Mix the diluted reagent from the previous step, vortex gently, and incubate for 5–10 min at room temperature (*see* Note 6). If RNA is used, you may perform this step on ice to avoid chemical and enzymatic degradation.
4. Wash the cells three times with serum-free medium.
5. Add the liposome complex, and incubate the cells at 37°C in a CO_2 incubator (5–10% CO_2) for 3–6 h. In general, transfection efficiency increases with time, although after 8 h, toxic conditions may develop.
6. Add 3 mL of medium with 20% of serum (the serum is dependent on the cell type; fetal calf serum may be used).
7. Incubate the cells for 24–48 h at 37°C in a CO_2 incubator.
8. Harvest the cells, and assay for gene activity (*see* Section 3.6.).

3.4. Promoter Expression Systems for Endogenously Expressed Ribozymes

Endogenous delivery involves the expression of an antisense RNA or a ribozyme from a DNA template permanently maintained within the cell. Examples of expression systems are given in Fig. 1, and have recently been reviewed elsewhere *(15)*. The reader should consider the following:

1. Expression of these molecules can be directed by polymerase II (Pol II) or polymerase III (Pol III) promoters. Pol II promoters include those promoters of viral origin, the long-terminal repeat (LTR) promoter sequences of retroviruses, or cellular promoters, such as the β-actin promoter.
2. Use of a strong promoter is often desirable, but high-level expression may be difficult to achieve. Tandem repeats of the antisense or ribozyme genes, under the control of the same promoter, may help to alleviate this obstacle by increasing the effective concentration of each transcript. Inducible, repressible, or tissue-specific promoters can be used to confer temporal, cell-type, and cell-specific expression, and may temper other problems, such as cellular toxicity generated by high levels of expression within the cells.
3. Endogenous expression from a Pol II promoter, with the exception of a few specialized cases, such as the human U1 snRNA Pol II promoter, necessitates a

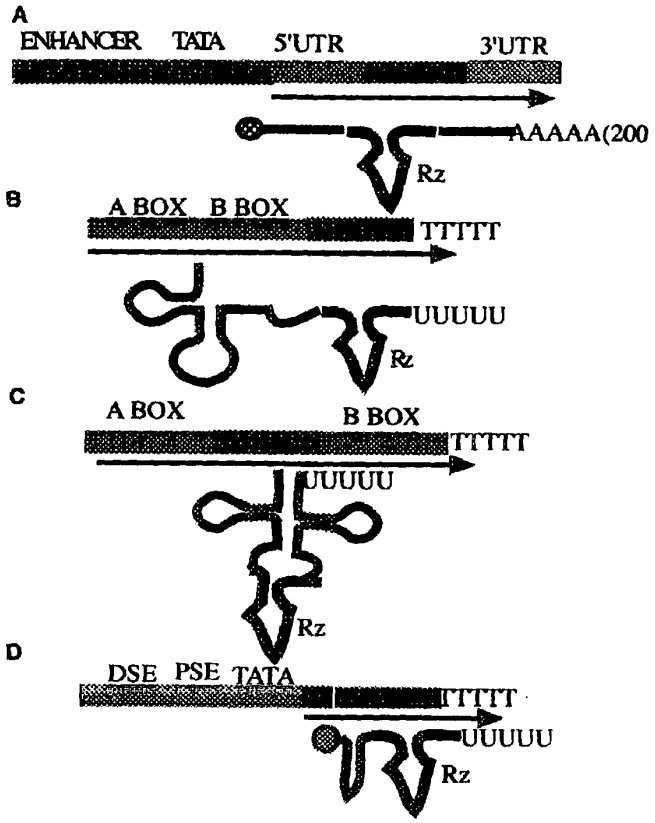

Fig. 1. Promoter systems for the intracellular expression of ribozymes. In (A)–(D), the top line depicts the gene encoding the ribozyme, and the transcript derived from that gene is illustrated beneath it. (A) depicts a typical RNA Pol II-type promoter for driving ribozyme transcripts. An important consideration in utilizing a Pol II system is that the ribozyme transcript will contain varying lengths of 5'- and 3'-appended sequences, including the poly (A) tract. (B) and (C) depict RNA Pol III promoter cassettes derived from a tRNA gene. The ribozyme replaces part of the pseudouracil stem loop and aminoacyl-acceptor stem (B), or is inserted into the anticodon loop (C). In both cases, intact A and B boxes are required for expression. A stretch of five uracils terminates transcription, and the transcript is not capped. (D) Shows a representation of the mammalian U6 small nuclear RNA (snRNA gene) promoter. This is a Pol III promoter, but unlike the tRNA gene, the promoter regulatory elements lie upstream of the mature coding sequence. The ribozyme construct is positioned immediately after the capping signal (a small stem loop structure). Transcription termination is signaled by a region of five uracils. DSE represents the distal sequence element, and PSE the proximal sequence element.

polyadenylation signal, which allows addition of a poly (A) tail. This, along with the 5'-m^{7}GpppG cap, common to Pol II transcripts, may prolong the intracellular half-life of the RNA molecules.

4. Expressing ribozyme molecules under the control of Pol III promoters affords additional advantages: Pol III-driven gene expression seems to occur at high levels in all tissues and cell types. The sizes of Pol III-transcribed genes are smaller, presenting more defined transcripts.
5. Other expression strategies are possible. For instance, an snRNA transcription unit that incorporates portions of the snRNA structural sequence and protein binding sites could be used. This can facilitate targeting to the nucleus. Another option is to insert a ribozyme into the acceptor arm or the anticodon loop of a tRNA gene, which has resulted in higher levels of expression and stability of the ribozyme *(16)*. However, this tRNA expression system has been shown to alter posttranscriptional processing and cellular transport.

3.5. Viral Vectors

Although the ribozyme or antisense expression vector can be delivered with liposomes, integration of the foreign DNA into the host genome does not occur with a high frequency. More promising and efficient technologies employ viral vectors.

Different viral vectors have the capacity to infect a variety of cell types with high efficiency. Several classes of viral vectors are being exploited for delivery of genes in vivo, including DNA (adenoviruses, herpesvirus, adeno-associated virus [AAV]) and RNA retroviruses. General concerns persist with the use of viral pathogens such as residual infectivity, toxicity, and rescue of infectivity by recombination. Additionally, each viral vector has its own set of advantages and disadvantages, which ultimately dictate its use in a specific application.

To date, the most extensively utilized viral vectors have been retroviruses. This class of viruses can infect a wide variety of cell types resulting in long-term persistence as a consequence of integration into the host chromosome. However, the integration process requires cell replication, thereby restricting retroviral use to actively dividing cells. Other potential concerns are low vector titers, lack of specific integration sites, the possibility of activating proto-oncogenes, and the potential for infectious helper virus rescue owing to recombination. Nonetheless, retroviruses possess properties for efficient and effective in vivo delivery, and are currently the method of choice.

An important consideration in expressing ribozymes from a retroviral vector is the positioning of the ribozyme transcription unit. In Fig. 2 A–D, several examples of ribozyme expression from a retroviral vector are illustrated. The most popular retroviral backbones utilize the amphotropic retroviral vectors developed by Miller and Rossman *(17)*. These have been engineered with convenient cloning sites in both the U3 regions of the LTRs and within the remnants of viral genes. If cloning of ribozyme genes into retroviral vectors is the method of choice, it is strongly encouraged that several modes for ribozyme

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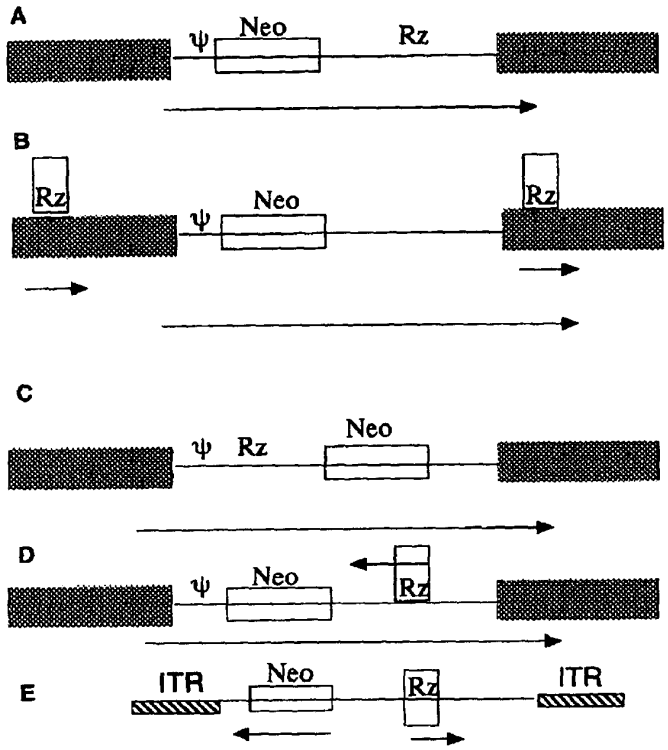

Fig. 2. Examples of viral vector constructs for ribozyme delivery and expression. **(A)–(D)** depict different versions of retroviral vectors. In (A) and **(C)**, the ribozyme (Rz) is expressed as part of the retroviral LTR transcript. In **(B)**, the ribozyme is driven by a Pol III promoter that is inserted as a double copy in the U3 region of the LTRs of the viral vector. In this construct, the direction of transcription is the same as that of the viral LTR promoter. In (D), the Pol III-ribozyme construct is transcribed in the opposite direction to the viral LTR. In **(E)**, expression of a ribozyme from an AAV is depicted. The ITRs have weak promoter function, but are generally not useful for expressing inserted genes. The ribozyme can be transcribed in either orientation using Pol II or Pol III transcriptional units. The arrows represent the direction and extent of transcription. Neomycin phosphotransferase (Neo) and retroviral packaging signals (ψ) are indicated.

transcription be tested. Packaging cell lines for amphotropic retroviral vectors, such as the NIH 3T3-derived PA317 cell system, are available through the ATCC and individual investigators. It is beyond the scope of this protocol to go into packaging methodologies. Those interested in pursuing retroviral-mediated gene transfer should consult some of the original manuscripts, which clearly describe the methodologies used for packaging and transduction *(17,18)*.

Another promising vector system for ribozyme gene delivery is AAV, which has been successfully used to transduce antisense RNA inhibitory to HIV infection *(19)*. The AAVs are nonpathogenic, integrating viruses that require helper viruses for replication of their genome. The AAV vector can exist autonomously at high copy number within a cell and can integrate into the host chromosomes as well. An example of an AAV vector useful for ribozyme expression is presented in Fig. 2E. As is the case for retroviral vectors, both Pol II and Pol III promoters can be utilized for ribozyme expression in AAV vectors. Coinfection with a helper virus, such as adenovirus, is required for productive infection *(19,20)*. Molecular clones of the AAV genome, such as that depicted in Fig. 2E, are infectious following transfection into helper-virus-infected cells *(20)*. The packaged DNA is single-stranded, and only the 145-base-long palindromic repeats or inverted terminal repeats (ITRs) are required for efficient packaging in the appropriate packaging cell line. In addition to the ribozyme transcriptional unit, it is useful to incorporate a selectable marker, such as the neomycin phosphotransferase gene. Those interested in using AAV as a vector for ribozyme delivery should consult the published protocols *(19,20)*.

3.6. In Vivo Assays for Ribozyme Function

For in vivo analyses, standard techniques are performed at the levels of RNA (Northern blots, primer extension, PCR), and Western blots or direct protein assays. Assays based on virus titer, reduction of infectivity, and proviral DNA are good indicators of viral inhibition. The RNA analyses should reveal a reduced amount of the targeted RNA and, in some cases, the presence of cleavage products. However, since RNA analysis is not by itself conclusive evidence for ribozyme-mediated cleavage, a mutant, noncleaving ribozyme should always be used as control to establish that any effect seen in vivo is a result of a specific ribozyme activity.

4. Notes

1. Reagents functionally similar to the Lipofectin agent, such as Lipofect ACE (Gibco/BRL), Transfectase (Gibco/BRL), Lipofect-AMINE (Gibco/BRL), Transfectam RM (Promega, Madison, WI), and DOTAP (Boehringer-Mannheim Corp.), can all be used in this procedure.
2. To produce radiolabeled transcripts use only 0.01–0.5 m*M* nonradioactive UTP, and 10 μCi of [α^{32}P]UTP (3000 Ci/mmol). Over 90% of the radioactivity can be incorporated into the transcripts.
3. The addition of spermidine increases the efficiency of the reaction. This step should be performed at room temperature to avoid DNA precipitation.
4. In vitro transcription can yield RNA up to 50 times the amount of DNA template utilized in the reaction.

5. For kinetic determinations, you may incubate a constant concentration of substrate (around 10 n*M*) with increasing amounts of ribozyme (beginning with two-fold molar excess of ribozyme) for a fixed time.
6. Prepare this complex in a polystyrene tube, because it can stick to polypropylene.

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References

1. Taylor, N. and Rossi, J. J. (1991) Ribozyme mediated cleavage of an HIV-1 gag RNA: The effects of non-targeted sequences and secondary structure on ribozyme cleavage activity. *Antisense Res. Dev.* **1**, 173–186.
2. Leonetti, J. P., Degols, G., and Lebleu, B. (1990) Biological activity of oligonucleotide-poly (L-lysine) conjugates: Mechanism of cell uptake. *Bioconjugate Chem.* **1**, 149–153.
3. Boutorin, A. S., Guskova, L. V., Inanova, E. M., Kobetz, N. D., Zarytova, V. F., Ryte, A. S., Yurchenko, L. V., and Vlassov, V. V. (1989) Synthesis of alkylating oligonucleotide derivatives containing cholesterol or phenazinium residues at their 3'-terminus and their interaction with DNA within mammalian cells. *FEBS Lett.* **254**, 129–132.
4. Letsinger, R. L., Zhang, G., Sun, D. K., Ikeuchi, T., and Sarin, P. S. (1989) Cholesteryl-conjugated oligonucleotides: synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture. *Proc. Natl. Acad. Sci. USA* **86**, 6553–6556.
5. Wu, G. Y., Wilson, J. M., Shalaby, F., Grossman, M., Shafritz, D. A., and Wu, C. H. (1991) Receptor mediated gene delivery *in vivo*. *J. Biol. Chem.* **266**, 14,338–14,342.
6. Abromova, T. V., Blinov, V. M., Vlassov, V. V., Gorn, V. V., Zarytova, V. F., Ivanova, E. M., Konevets, D. A., Plyasunova, O. A., Pokrovsky, A. G., Sandahchiev, L. S., Svinarchuk, F. P., Starostin, V. P., and Chaplygina, S. R. (1991) Anti-HIV activity of antisense oligonucleotides bearing lipophilic and alkylating groups at the 5' terminus. *Nucleotides and Nucleosides* **10**, 419–422.
7. Kaneda, Y., Kunimitsu, I., and Uchida, T. (1989) Increased expression of DNA co-introduced with nuclear protein in adult rat liver. *Science* **243**, 375–378.
8. Sugawa, H., Uchida, T., Yoneda, Y., Ishiura, M., and Okada, Y. (1985) Large macromolecules can be introduced into cultured mammalian cells using erythrocyte membrane vesicles. *Exp. Cell Res.* **159**, 410–418.
9. Curiel, D. T., Wagner, E., Cotten, M., Birnstiel, M. L., Agarwal, S., Li, C. M., Loechel, J., and Hu, P. C. (1992) High efficiency gene transfer mediated by adenovirus coupled to DNA-polylysine complexes. *Hum. Gen. Ther.* **3**, 147–154.
10. Bertrand, E., Fromont-Racine, M., Pictet, R., and Grange, T. (1993) Visualization of the interaction of a regulatory protein with RNA *in vivo*. *Proc. Natl. Acad. Sci. USA* **90**, 3496–3500.

11. Freier, S. M., Kierzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M. H., Neilson, T., and Turner, D. H. (1986) Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl. Acad. Sci. USA* **83,** 9373–9377.
12. Wright, S. and Huang, L. (1989) Antibody directed liposomes as drug delivery vehicles. *Adv. Drug Delivery Rev.* **3,** 343–390.
13. Ho, R. J. Y., Rouse, B. T., and Huang, L. (1987) Target-sensitive immunoliposomes as an efficient drug carrier for antiviral activity. *J. Biol. Chem.* **262,** 13,973–13,978.
14. Alving, C. R. (1988) Macrophages as targets of liposome-encapsulated antimicrobial agents. *Adv. Drug. Delivery Rev.* **2,** 107–128.
15. Rossi, J. J. (1995) Controlled, targeted, intracellular expression of ribozymes: progress and problems. *Trends Biotechnology* **13,** 301–306.
16. Cotten, M. and Birnstiel, M. L. (1989) Ribozyme mediated destruction of RNA *in vivo*. *EMBO J.* **12,** 3861–3866.
17. Miller, A. D. and Rossman, G. J. (1989) Improved retroviral vectors for gene transfer and expression. *Biotechniques* **7,** 980–990.
18. Miller, A. D. and Buttimore, C. (1986) Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell Biol.* **6,** 2895–2902.
19. Chatterjee, S., Johnson, P. R., and Wong, K. K., Jr. (1992) Dual target inhibition of HIV-1 *in vivo* by means of an adeno-associated virus antisense vector. *Science* **258,** 1485–1488.
20. Chatterjee, S. and Wong, K. K., Jr. (1993) Adeno-associated viral vectors for the delivery of antisense RNA. *Methods: A Companion to Methods Enzymol.* **5,** 51–59.